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PRINCIPAL INVESTIGATOR: Ralph D. Sanderson, Ph.D.

CONTRACTING ORGANIZATION: University of Arkansas for
Medical Sciences
Little Rock, Arkansas 72205

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6. AUTHOR(S)
Ralph D. Sanderson, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Arkansas for Medical Sciences
Little Rock, Arkansas 72205

E-MAIL:
SandersonRalphD@exchange.uams.edu

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13. ABSTRACT ***(Maximum 200 Words)***

Heparan sulfate proteoglycans (HSPGs) are a new class of tumor suppressors. The focus of this project is to test novel proteoglycan based therapies for the treatment of breast cancer. In the first objective, the ability of neoproteoglycans (nPGs) to mimic the anti-tumor activities of naturally occurring proteoglycans is evaluated. In the first year we have successfully produced nPGs with different glycosaminoglycan chains coupled to a protein scaffold. SDS-PAGE analysis confirms nPG production and indicates contamination with glycosaminoglycan chains not coupled to protein. nPG is isolated by size exclusion chromatography and the resulting fractions evaluated for anti-cancer activity. Surprisingly, the low molecular weight fractions, containing glycosaminoglycan chains only, reduce breast cancer cell viability while the nPG containing fractions do not. Therefore, neoglycans, possibly composed of glycosaminoglycan chains coupled to each other, were produced and these reduce breast cancer cell viability. In the second objective, a gene therapy approach is tested utilizing the HSPG gene syndecan-1. Tagged full length and truncated human syndecan-1 genes have been constructed and initial studies in mice completed, with inconclusive results. This project is the first attempt to use HSPG genes therapeutically and to produce nPGs and neoglycans for anti-cancer therapy. Results of this first year of work highlight the potential of this strategy.

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INTRODUCTION:

Current data strongly support the idea that heparan sulfate proteoglycans (HSPGs) represent a new class of tumor suppressors. When syndecan-1, a HSPG, is lost from the surface of mammary epithelia, the cells lose their epithelial morphology, invade collagen gels and exhibit characteristics of neoplastic growth. When transfected with the cDNA for syndecan-1, transformed mammary epithelial cells regain the epithelial morphology and lose neoplastic growth characteristics. Remarkably, addition of purified intact syndecan-1 ectodomain to tumor cells inhibits growth in culture and induces apoptosis. These growth inhibitory activities are not exclusive to syndecan-1 because the HSPGs glypican-1 and betaglycan have similar affects on tumors. The work of this proposal focuses on the development of novel proteoglycan-based therapies that will stop the growth and perhaps kill breast tumor cells. Because the anti-tumor effects are not specific to any one HSPG we propose in the first objective to construct neoproteoglycans (nPGs) that will potentially mimic the activities of naturally occurring HSPGs. nPGs will be produced by coupling heparin or other glycosaminoglycan chains to human serum albumin (HSA). These molecules will be characterized and analyzed for anti-tumor effects when added to cells in culture or injected directly into established tumors growing in the mammary fat pad of nude mice. The second objective of this project is to transfer the syndecan-1 gene directly in breast tumors growing in nude or SCID mice. Following gene transfer the effects of syndecan-1 expression on tumor growth will be analyzed. This project represents the first attempt to use HSPGs as anti-cancer therapy.

BODY:

Task 1: To produce and characterize neoproteoglycans (nPGs) and test their efficacy in inhibiting tumor growth in vitro and in vivo (months 1-36).

- Prepare and characterize nPGs using various glycosaminoglycans (heparin, heparan sulfate, chondroitin sulfate) and HSA (1-6 months).

- Test nPGs in cell culture for their ability to inhibit tumor cell growth (months 7-18).

Heparin and chondroitin sulfate are covalently coupled to HSA by carbodiimide condensation reaction generating nPGH and nPGCS, respectively. Following the coupling reaction, the preparations are buffer exchanged into water by extensive spin column filtration or dialysis. To confirm nPG production, reaction products are separated by SDS-PAGE on 12% gels and the protein content visualized by coomassie staining followed by alcian blue staining to reveal glycosaminoglycan content (Figure 1). Attachment of glycosaminoglycan chains to HSA should generate a heterogeneous population of molecules due to the variation in the number of glycosaminoglycan chains incorporated and the size of the individual glycosaminoglycan chains. The 66 kDa HSA band is not visible by coomassie staining suggesting that coupling to glycosaminoglycan

chains has occurred (Figure 1, Panel A, Lanes 3 and 5). High molecular weight nPGs and low molecular weight unincorporated glycosaminoglycan chains are apparent following alcian blue staining (Figure 1, Panel B, Lanes 3 and 5). Polysaccharide lyase treatment removes glycosaminoglycan chains and reveals the 66 kDa protein scaffold of each nPG (Figure 1, Panels A, and B, Lanes 4 and 6).

Several breast cancer cell lines including MCF-7, MDA-MB-231, MDA-MB-435, and MDA-MB-436 were treated with nPGH and nPGCS and cell viability was determined by MTT assay. Results from a representative experiment demonstrate that nPGH and nPGCS treatment of breast cancer cell lines significantly reduces cell viability. Simply mixing HSA and glycosaminoglycan chains does not affect cell viability, confirming that the carbodiimide coupling reactions is required for activity.

The carbodiimide condensation reaction products include nPG and excess glycosaminoglycan chains (Figure 1, Panel B). Because varying number and sizes of glycosaminoglycan chains are added to the HSA scaffold (66 kDa) the resulting nPG is a heterogeneous population of molecules greater than 66 kDa in size. The remaining low molecular weight alcian blue staining material is excess glycosaminoglycan chains that could presumably block nPG activity. Therefore, the reaction products were separated by size exclusion chromatography using a Superdex 200 column. Fractions were collected and buffer exchanged. SDS-PAGE analysis shows fractions containing nPGH (Figure 3, Panel A, lanes 33-34), nPGH and heparin not bound to protein (Figure 3, Panel A, lanes 35-41), and lower molecular weight heparin only (Figure 3, Panels A and B, lanes 37-52). Each fraction was evaluated in a cell viability experiment. Surprisingly, the low molecular weight fractions 47-52 that do not contain a protein scaffold greatly reduced cell viability while the nPG containing fractions 33-41 did not (data not shown). With this result in mind, coupling reactions were set up with glycosaminoglycan chains only and the products tested for the ability to reduce the cell viability of several breast cancer cell lines including MCF-7, MDA-MB-435, MDA-MB-436, and MDA-MB-231. The results are shown in Figure 4, Panels A – D. Neoglycans, possibly composed of glycosaminoglycan chains coupled to each other, very effectively reduce breast cancer cell viability whereas unmodified glycosaminoglycan chains do not. Therefore, a protein scaffold is not required for the production of molecules that actively lower breast cancer cell viability.

Task 2: To determine if transfer of the syndecan-1 gene into breast cancer tumors is a viable therapeutic approach (months 1-36)

- Prepare liposomal and retroviral vectors for transfer of the syndecan-1 gene (months 1-12).

- Inject vectors into tumors growing in nude mice, confirm syndecan-1 expression and determine effects on tumor growth and viability (months 18-36).

Syndecan-1 expression was characterized on several breast cancer cell lines including MCF-7, MDA-MB-231, MDA-MB-435, MDA-MB-436, and Hs578t. Each of these cell lines does express syndecan-1 on the cell surface. Therefore, for the purpose of distinguishing native syndecan-1 from syndecan-1 expressed from transferred gene cassettes a c-myc tag was incorporated into the human syndecan-1 gene construct by site directed mutagenesis. Primers for the production of three syndecan-1 gene cassettes including a full-length cassette and two truncated constructs have been engineered and used to PCR amplify the appropriate DNA fragments. Expression of the truncated cassettes will generate syndecan-1 that will be secreted instead of found on the cell surface. The three syndcan-1 constructs have been cloned in plasmid vectors.

In vivo experiments to test the effect of increased syndecan-1 expression on tumor growth have begun. MDA-MB-231 breast cancer cells were injected into the mammary fat pad of SCID mice and tumors were established. Plasmid DNA only or plasmid DNA containing the full-length human syndecan-1 gene cassette was electroporated into tumors two times per week for several weeks. Tumor growth was measured every other day and at the end of the experiment the animals were sacrificed and the tumors excised and stained for syndecan-1 expression. The results showed no reduction in tumor size however, the tumors were initially very small and did not grow appreciably regardless of treatment. The data from the tumor staining was questionable due to technical error. The expression of the therapeutic gene could not be confirmed and therefore, this experiment is inconclusive.

KEY RESEARCH ACCOMPLISHMENTS:

Task 1.

- Neoproteoglycans have been prepared by carbodiimide condensation reaction.
- Neoproteoglycan production has been confirmed by SDS-PAGE analysis.
- Unseparated reaction products reduce breast cancer cell viability.
- Size exclusion chromatography separates nPG from excess glycosaminoglycan chains.
- Low molecular weight fractions reduce cell viability while high molecular weight fractions containing nPG do not.
- Neoglycans, composed of glycosaminoglycan chains only, reduce breast cancer cell viability.

Task 2.

- A c-myc tag has been added to the human syndecan-1 gene.
- Full length and two truncated human syndecan-1 gene cassettes have been PCR amplified and cloned.
- Treatment of breast cancer tumors in mice with the full-length human syndecan-1 gene has been attempted.

REPORTABLE OUTCOMES:

- A neoglycan manuscript is in preparation.
- A provisional patent entitled "Synthetic, Highly Charged Molecules and Uses Thereof" was filed on January 8, 1999 and the patent application filed January 7, 2000.

CONCLUSIONS:

The first objective to construct nPGs and test the efficacy in inhibiting the growth of breast cancer cells has yielded very interesting and surprising results. Following production, the nPGs were separated from excess glycosaminoglycan chains and fractions were evaluated for reduction in tumor cell viability. The results indicate that low molecular weight moieties devoid of protein greatly reduce cancer cell viability. Production and evaluation of neoglycans, carbodiimide condensation reaction products containing glycosaminoglycan chains and no protein, demonstrates that these molecules effectively reduce breast cancer cell viability whereas untreated glycosaminoglycan chains do not. Neoglycan production and evaluation as an anti-cancer therapy is completely novel and a very exciting outcome of the proposed work.

The second objective of the proposal is to transfer syndecan-1 gene constructs into breast cancer cells lines and tumors growing in mice to test the ability of this tumor suppressor gene to slow growth. Several different syndecan-1 gene cassettes have been produced during this year and the full-length syndecan-1 gene has been electroporated into established breast cancer tumors in mice. While the results of the in vivo study were inconclusive, we are confident that we have worked out the technical difficulties and will address this important question. This work represents the first attempt to use HSPGs and to construct nPGs and neoglycans for the purpose of anti-cancer therapy. After the first year of investigation the potential of this strategy is evident.

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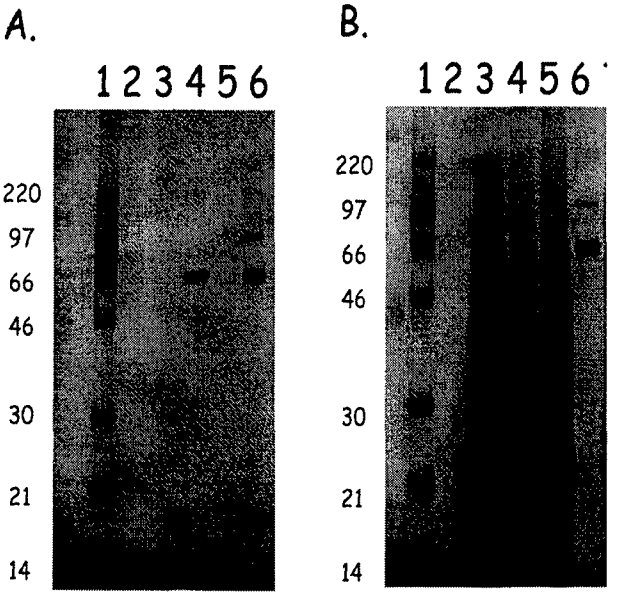

Figure 1. nPGs produced by carbodiimide condensation reaction. Both panels show SDS-PAGE separation of reaction products. Panel A is stained with coomassie blue revealing the protein content of the reactions. In Panel B the same gel is additionally stained with alcian blue to display the glycosaminoglycan content. Lanes 1 contain molecular weight standards. Lanes 3 and 4 contain nPGH untreated and heparinase treated, respectively. Lanes 5 are the intact nPGCS and lanes 6. are chondroitinase treated nPGCS.

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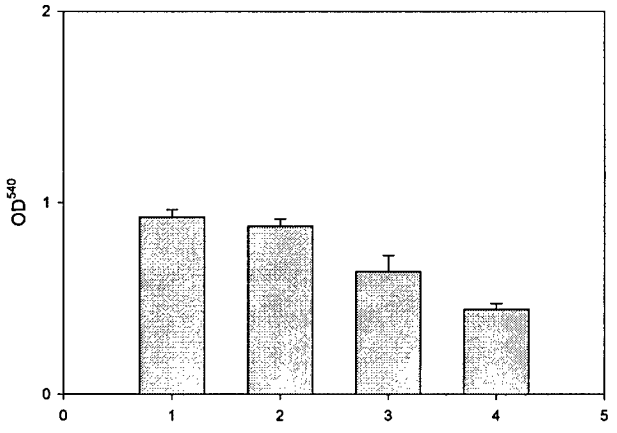

Figure 2. nPGH and nPGCS reduce breast cancer cell viability. MCF-7 breast cancer cells were treated with media only (column 1), a mixture of heparin and HSA (column 2), nPGCS (column 3) and nPGH (column 4). Following a 48 hour incubation at 37°C, cell viability was determined by MTT assay. Experiments were performed in triplicate.

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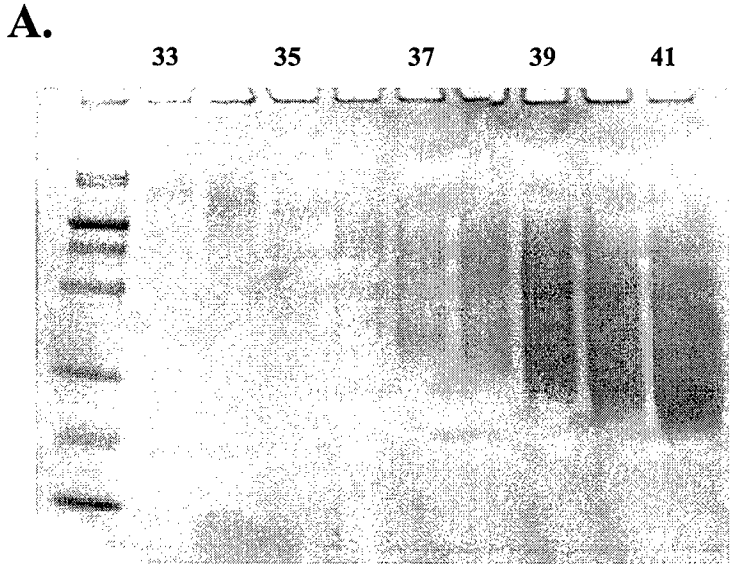

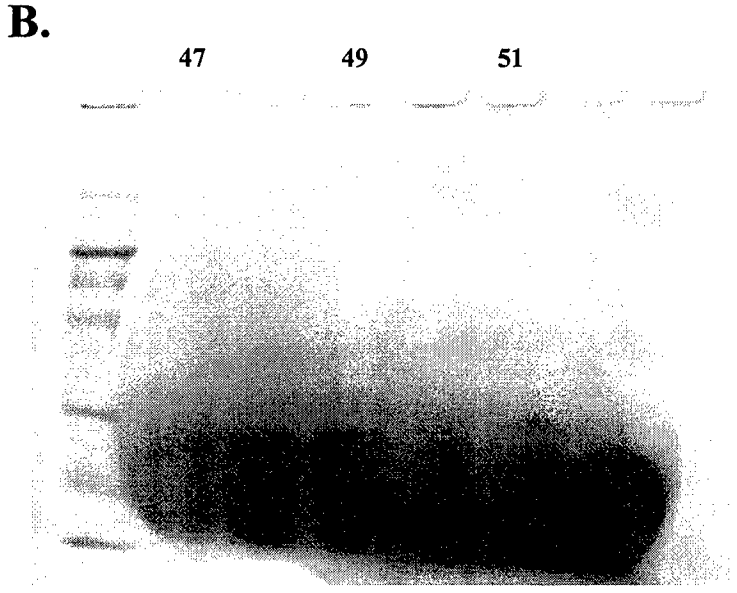

Figure 3. nPGH is separated from low molecular weight glycosaminoglycan chains by size exclusion chromatography. Fractions are visualized by SDS-PAGE and alcian blue staining. Fractions are numbered along the top of each gel.

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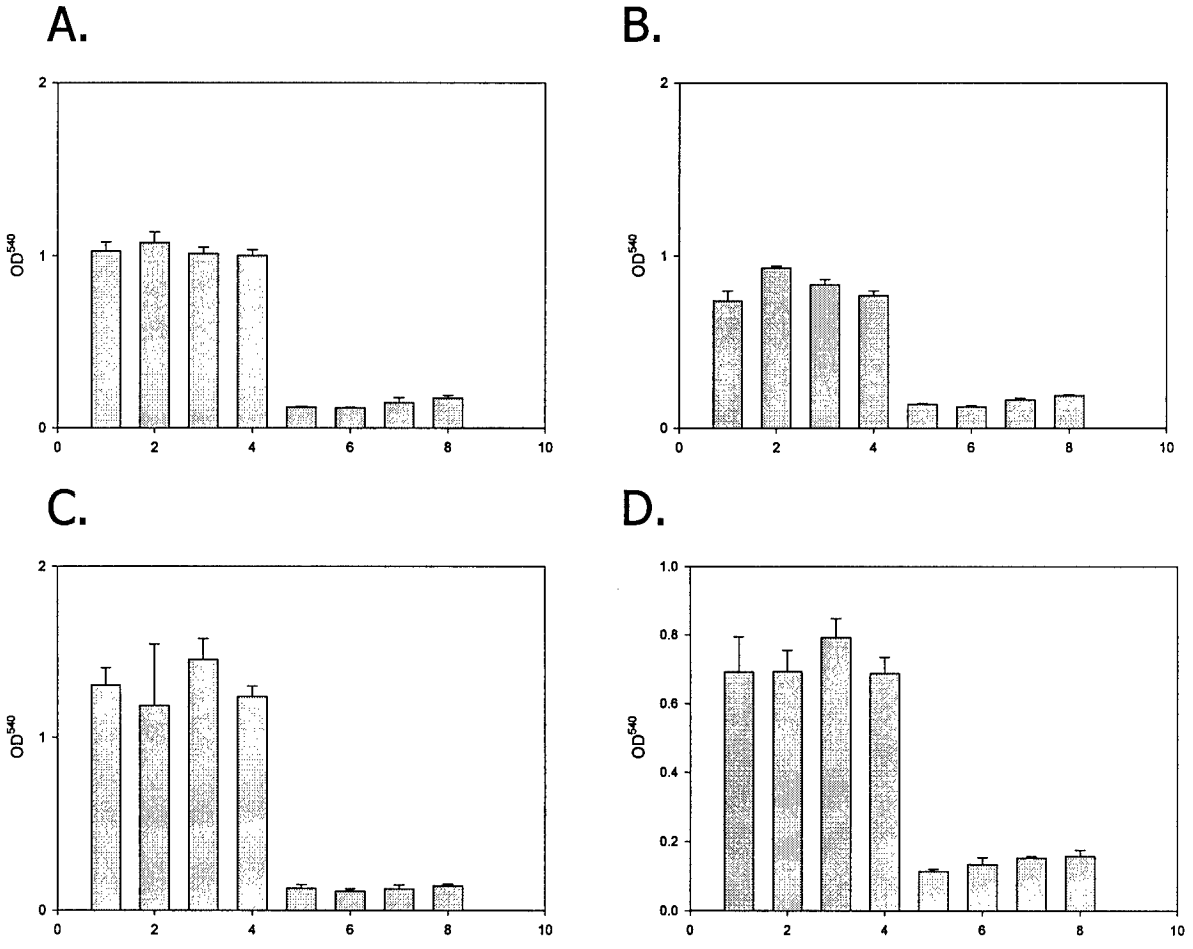

Figure 4. Neoglycans reduce breast cancer cell viability. MCF-7 (Panel A.), MDA-MB-231 (Panel B.), MDA-MB-435 (Panel C.), and MDA-MB-436 (Panel D.) breast cancer cells were treated in triplicate with media only (columns 1.), dexamethasone (columns 2.), heparin (columns 3.), chondroitin sulfate (columns 4.), heparin neoglycan (columns 5. – 6.), and chondroitin sulfate neoglycan (columns 7. – 8.) for 48 hours at 37° C. Cell viability was determined by MTT assay.

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US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
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FORT DETRICK, MARYLAND 21702-5012

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